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14. ABSTRACT
Rationale: Multiple myeloma (MM) is an incurable malignancy derived from antibody secreting B lineage plasma cells. MM is the second most common malignancy in the United States and accounts for 1% of cancer deaths. Despite recent advances, the 5-year survival rate in patients with MM is less than 40% MM responds well to chemotherapy and remissions occur in that majority of MM patients, but all patients eventually relapse and die from progressive disease within 6 years. If the residual post-remission cells of their activation to progressive disease could be disrupted with novel targeted therapies. It would have a significant impact on the care and treatment of MM patients, particularly male veterans who are at 51% increased risk of MM compared to general public.

15. SUBJECT TERMS- none provide

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1. Introduction:

Multiple myeloma is the second most common hematological cancer in the world. It is characterized by accumulation of malignant plasma cells in the bone marrow, osteolytic lesions and monoclonal immunoglobulins in blood and/or urine (Antonio, 2011). While understanding of the mechanisms of drug resistance in MM is limited, interactions with the bone marrow microenvironment are a major contributing factor (Masahiro A, 2011). Thus, novel agents targeting the tumor vasculature and microenvironment are needed. One approach is to exploit the membrane glycoproteins including integrins that mediate stromal interactions. Integrins are heterodimer adhesive receptors expressed by virtually all cell types, including cancer cells (Hynes, 2002; Desgrosellier JS 2010). Several integrins are expressed by MM cells, predominantly the α4, α5, αv and the β1 subunits. The α4β1 integrin (CD49d or VLA-4) is expressed by both normal and malignant plasma cells (Pals ST, 2007) and is over-expressed in drug-resistant MM cells (Damiano JS, 2010). Integrins are critical to tumor angiogenesis, cell adhesion, and migration (Ruoslahti *et al.*, 1996). Adherent integrin-stromal interactions also enhances the proliferation of MM cells (Ria, R, 2002.). MM still remains an incurable disease and disease relapse occur in the vast majority of MM patients.

Myc genes encode basic helix–loop–helix–leucine zipper (bHLHZip) transcription factors that heterodimerize with their partner protein Max to bind DNA, regulate target gene expression, and modulate numerous biological functions (Amati et al., 1993; Crouch et al., 1990, 1993; Evan et al., 1992; Freytag et al., 1990; Smith et al., 1990). The Myc pathway plays a central role in the evolution of MGUS into MM (Anguiano A, 2009). [ALSO PLEASE SEE/CITE: http://www.jci.org/articles/view/61188 and PMID 22806891] The c-Myc protein is up-regulated in a large fraction of human cancers yet

remains a challenging target for drug discovery (Hermeking, H. 2003, Prochownik, E. V 2004' Darnell, J. E 2002, Gibbs, J. B. 2000). Several small-molecule inhibitors of the c-Myc-Max interaction have been reported (Kiessling, A2006, Mo, H.; Henriksson, 2006, Xu, Y 2006, Berg, T 2002, Bagnasco, L 2007, Pescarolo, M. P 2001, Yin, X. 2003,), but none have yet been validated in clinical trials. Max inhibitors could be explored for their therapeutic potentials for various diseases overexpressing Myc including cancers such as multiple myeloma (Toril H, 2012).

Nanoparticles are promising carriers for anticancer agents that allow for selective drug delivery and increased drug levels at target sites. (Temming, Kai, 2005), Here, we investigated the effects of integrin-targeted nanoparticles as Myc drug delivery vehicles to MM cells. The selectivity of these targeted NPs was achieved by exploiting the presence of the Integrins $\alpha_v\beta_3$ and $\alpha_4\beta_1$, which are known to be linked to both tumor angiogenesis and metastasis in MM cells.

2. Keywords: Myeloma, Cancer, Nanoparticles, Nanotechnology

3. Overall Project Summary:

Myc Drug and Prodrug are cytotoxic for MM cells: The effects on the cellular growth and viability were determined using murine and human MM cell lines. A significant decrease in cell viability was observed following treatment with the Myc Prodrug in comparison to the myc drug at equimolar concentrations in all MM cells lines at 24 Hrs **(Figure 2 A)**. The results were confirmed by PE Annexin V staining. 92%, 91% and 92% of cells treated with Prodrug for 24 hrs were observed to be PE Annexin V positive, suggesting that they were in end stage apoptosis or already dead **(Figure 2B),** compared to 19%, 31% and 31% treated with the Myc drug in H929, U266 and 5TGM1 cells respectively . Increased cytotoxicity observed with prodrug versus base compound was expected due to increased hydrophobicity caused by addition of hydrocarbon tail. We developed nanoparticle envelopes to target the Myc prodrug because this hydrophobic compound would not be workable for animal or human studies.

Expression levels of Integrins αvβ3 and α4β1 on MM cells: To determine whether the target (Integrin αvβ3 and α4β1) specific delivery of the Prodrug within the NPs is correlated with quantitative changes in expression of nanoparticle integrin targets, we examined integrin subunit (α4 and αv) expression in human and mouse MM cell lines using immunoblots. Integrin αv was expressed only in H929 and U-266 cells at protein levels **(Figure 3A)** whereas Integrin α4 was abundantly expressed in the all the MM cell lines used (**Figure 4A)**. Manganese {Mn^{2+}) treatment induces "inside-out" signaling and conformational activation of integrin receptors which significantly changes epitope availability (Hu P, 2012). Expression of Integrin αvβ3 on cells was also determined using flow cytometry with a species-specific antibody for both Mn activated and non-

activated human MM cell lines. Interestingly, only the cell lines expressing the protein for Integrin αv **(Figure 3A)** (H929 and U-266) showed almost 40% of cells **(Figure 3B).**

Specificity of integrin targeting of nanoparticles to MM cells correlates with target expression. To verify that the specificity and binding of these nanoparticles to the cells depends upon the expression levels of the integrins, human H929 and U-266 cells expressing high levels of both the Integrins, αvβ3 and α4β1 were used. These MM cells were then treated Rhodamine Labeled Integrin Targeted (Integrin αvβ3 and α4β1) and non-Targeted Nanoparticles. As postulated, the Integrin expressing cells bound to Integrin targeted NP with higher affinity than the non-targeted counterparts, with the integrin αvβ3 Tg NP labeling 64% (H929) and 54% (U266) cells and Integrin α4β1 Tg NP labeling 65.9% (H929) and 65.5 (U266) cells. **(Figure 3C & 4B)**. We next sought to determine whether the 5TGM1 murine cell line expressed these integrins, and we found 5TGM1 highly expressed Integrin α4 (89%) **(Figure 4D)** and very low levels of integrin αv (8.1%) **(Figure 3D),** which was further confirmed by binding of rhodamine-labeled counterparts of α4β1 targeted NPs to these cells **(Figure 4C).**

α4β1 targeted-**NPs are cytotoxic for myeloma cell lines**

We evaluated the cytotoxicity of the integrin-targeted NPs on myeloma cell lines. Integrin αvβ3 Tg NPs weakly inhibited the growth of KMS-11 and 5TGM1, which was expected as integrin αvβ3 was not present at significant levels in both the cell lines **(Figure 5C & D)**. In contrast, Tg (Integrin αvβ3 and α4β1) NPs significantly inhibited the growth of high-expressing U266 and H929 cell lines at equimolar concentrations **(Figure 5A & B)**. Together, these data demonstrate that the integrin-Tg NP specifically

inhibited the growth of myeloma cell lines in relation to the expression levels of the target integrin on the surface of the myeloma cells. Control experiments performed with media, DMSO and Myc Drug yielded very similar results to those obtained with non-targeted NPs. Whereas, the efficacy of the myc prodrug in cell killing was significantly higher in all the cell lines irrespective of the Integrin expression levels. Therefore, using Tg NPs for the delivery of this prodrug showed cytotoxic effects with higher specificity depending on the Integrin expression of the cell line used.

4. Key Research Accomplishments:

1. We obtained approval including the local IACUC and DoD Office of Research Protection approval.
2. We performed in vivo efficacy studies of VLA-targeted Sn-2 Myc-inhibitor nanoparticles in mouse models of MM. We have collected blood and tissue for assays to correlate with overall visceral and bone tumor burden and estimate osteoclast and osteoblast activity and tumor burden.
3. We performed quantitative flow cytometry and histological assessments of bone marrow multiple myeloma tumor cell burden and collateral marrow hematopoietic and stromal populations in response to VLA- targeted Sn-2 Myc-inhibitor nanoparticles.

5. Conclusion:

We have completed initial testing of VLA-4-targeted, anti-Myc nanoparticles and have found specific and significant effects using myeloma cell lines. We are now proceeding to the characterization of these nanoparticles in pre-clinical mouse models of myeloma that we have "up and running" in the laboratory. We are currently investigating the effects of anti-Myc nanoparticles on tumor cells and normal tissues of mice.

6. Publications, Abstracts, and Presentations:

None

7. Inventions, Patents and Licenses:

None

8. Reportable Outcomes:

Figure 1

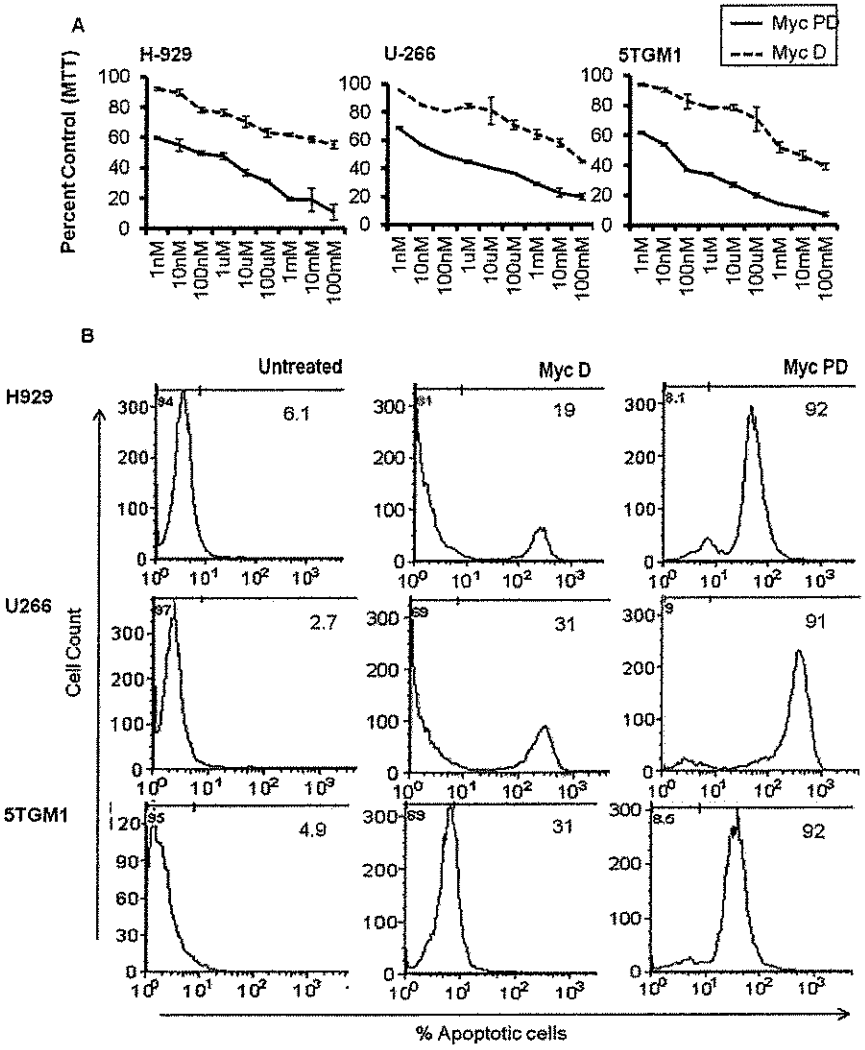
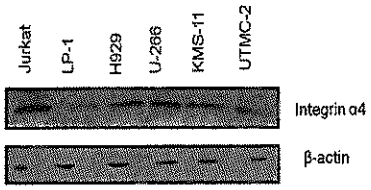


Figure 2

A.



B.

Cell lines	% α4β1 on cell surface	
	Non-Tg NP	VLA-4 Tg NP
Jurkat	3.6	77.6
H-929	6.2	65.9
U-266	1.7	65.5
KMS-11	2.06	53.32
UTMC-2	5.67	47.96
LP-1	0.17	5.27

C.

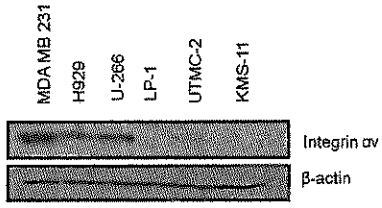
Cell line	% α4β1 on cell surface	
	Non-Tg NP	VLA-4 Tg NP
5TGMI	18	75

D

Cell line	% α4β1 on cell surface	
	Isotype control	Integrin α4
5TGMI	18	89

Figure 3

A.



B.

Cell lines	% αvβ3 on cell surface with LM609	
	w/0 activation	with activation
MDA-MB-231	15.5	44
H-929	4.79	43
U-266	4.54	40
KMS-11	1.2	4.6
UTMC-2	4.53	9.9
LP-1	0.43	1.5

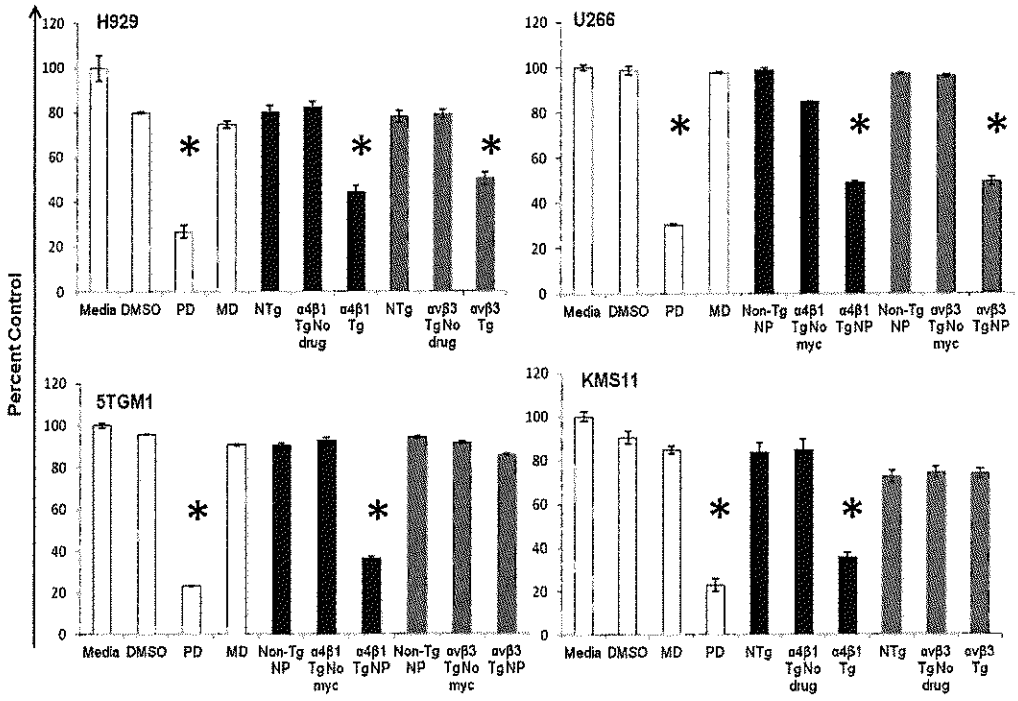
C.

Cell lines	% αvβ3 on cell surface of cells	
	Non-Tg NP	avb3Tg NP
MDA-MB-231	13	92
H-929	5.6	64
U-266	1.7	54
KMS-11	1.6	16
UTMC-2	2.1	3.3
LP-1	0.95	0.99

D

Cell line	% αvβ3 on cell surface	
	Isotype control	Integrin αv
5TGMI	0.62	8.1

Figure 4


Percent Control
H929
U266
5TGM1
KMS11
Media
DMSO
PD
MD
NTg
α4β1 TgNo drug
α4β1 Tg
αvβ3 TgNo drug
αvβ3 Tg
Non-Tg NP
α4β1 TgNo myc
α4β1 TgNP
αvβ3 TgNo myc
αvβ3 TgNP

9. Other Achievements:

None

10. References:

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Appendices: None